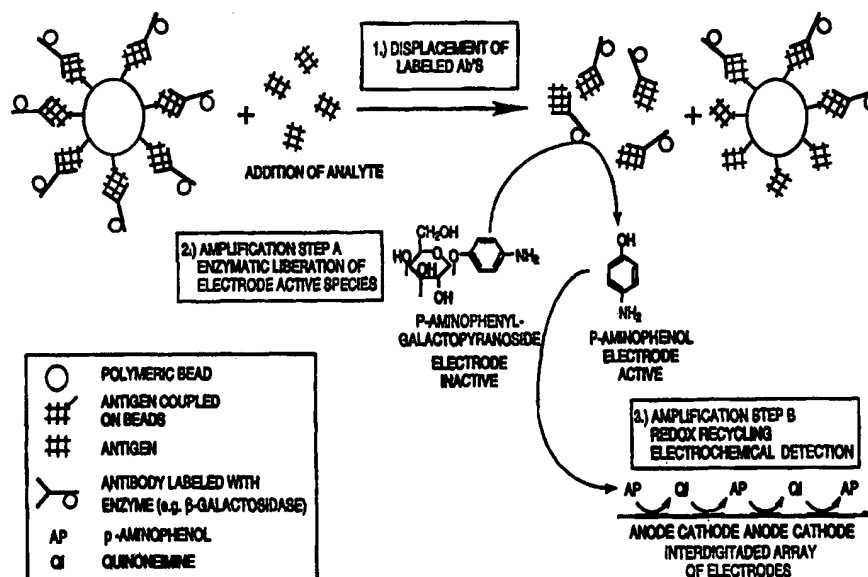




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(54) Title: ELECTROCHEMICAL REPORTER SYSTEM FOR DETECTING ANALYTICAL IMMUNOASSAY AND MOLECULAR BIOLOGY PROCEDURES



(57) Abstract

An immunochemical and molecular biologic endpoint reporter system in which reaction products, coupled to electrochemically active molecules susceptible to redox recycling or coupled to enzymes capable of proportional generation of said electrochemically active molecules, are detected and/or quantitated using amperimetry in conjunction with a silicon microchip possessing a closely spaced interdigitated array of noble metal electrodes.

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ELECTROCHEMICAL REPORTER SYSTEM FOR
DETECTING ANALYTICAL IMMUNOASSAY AND
MOLECULAR BIOLOGY PROCEDURES

5 This application claims benefit of United States
Provisional Application No. 60/055,466, filed August 12, 1997;
United States Provisional Application No. 60/055,759, filed
August 14, 1997 and United States Application Serial Nos.
09/105,538 and 09/105,539, filed June 26, 1998.

10 FIELD OF INVENTION

The present invention relates to an electrochemical
method and an associated microchip-based apparatus that can be
used to afford voltammetric or amperimetric detection for
monitoring immunochemical and/or molecular biology procedures.

15 BACKGROUND OF INVENTION

To assess the utility of any chemical reaction, whether
it be inorganic, organic or biochemical, the composition and
relative quantities of reactants and products must be
determined while the reaction is in progress or at its
20 equilibrium endpoint. One specific means of affecting such
monitoring utilizes biologic or non-biologic molecules capable
of binding to either reactant or product molecules in a
structure restricted manner. These analytic techniques are,
in general, referred to as immunochemical, in reference to the
25 selective recognition and binding capacity of immunoglobulins,
even though substances other than antibodies may serve as
recognition molecules. The terms receptor and ligand have
been used to more generally describe this area of analytic
art. Although diversely applied in basic organic and
30 biochemistry, these techniques have seen their most prolific
development in the field of clinical medicine, and relevant
generalized principles for measuring the progress of reactions

immunochemically, although applicable to many other scientific pursuits, can be illustrated using examples from this field. Here, a multitude of immunochemically formatted tests have been developed for measuring virtually any biologic molecule
5 of clinical importance. Such analytic procedures represent the cornerstones for laboratory studies in toxicology, endocrinology, immunology, serology, microbiology, and enzymology, to name but a few.

The most frequently utilized methods at present, are the
10 enzyme-linked immunosorbant assays (ELISAs). These procedures are applicable to a wide variety of fields such as biotechnology, environmental protection and public health. The performance of these conventional state of the art colorimetric methods of detection suffer from the infirmities
15 of having requirements for optical clarity, photomultiplication, signal digitalization or analog quantitation and transmission, viscosity or background chromogenic neutrality.

In the immunochemistry field, for example, enzyme
20 immunoassays (EIA) and, more particularly enzyme-linked immunosorbent assays (ELISA) are well known in the art and have become important and relatively cost-efficient tools of clinical laboratories for detecting traces of foreign substances, such as antigens or antibodies in body fluids and
25 tissues. (See, e.g. Immunoassay, Diamandis, E. and Christopoulos, T. eds., (1996); Clausen, J., Immunochemical Techniques for the Identification and Estimation of Macromolecules (Laboratory Techniques in Biochemistry and Molecular Biology) Vol. 1 (1989); Tijssen, P., Practice and
30 Theory of Enzyme Immunoassays (Laboratory Techniques in Biochemistry and Molecular Biology), (1985); Principles and Practice of Immunoassay, 2d ed., Price, C. and Newman, D., eds. (1997))

Such immunoassays, while generally reliable, depend on
35 sophisticated and extremely expensive optical processes to

report their results. Such optical processes are cumbersome because they are expensive, require a clean and unsoiled measurement chamber and their visually rendered signals prevent precise quantitation of results in a simple manner.

5 State of the art optical systems have several drawbacks, in that they generally require optical clarity, photo multiplication, signal digitalization or analog quantitation and transmission, as well as compatible viscosity and/or a neutral optical background. Transparent support media,

10 aqueous or otherwise, may become fouled or turbid and prevent or render difficult any accurate analyses utilizing optical reporters.

Attempts have been made to provide systems other than optical ones to detect antigens in body fluids. Duan, C. et

15 al., "Separation-Free Sandwich Enzyme Immunoassays Using Microporous Gold Electrodes and Self-Assembled Monolayer/Immobilized Capture Antibodies," Analytical Chemistry, 66/9:1369-77 (1994) discloses a separation-free system aimed at simplifying conventional immunoassay protocols

20 utilizing a gold-plated microporous membrane which serves as the solid phase for a noncompetitive sandwich-type immunoassay as well as a working electrode of an amperimetric detection system. A capture monoclonal antibody is covalently immobilized by a conventional chemical bonding agent at the

25 gold plated side of the membrane. A model analyte protein as well as an alkaline phosphatase labeled antibody are incubated simultaneously with the immobilized capture antibody. Surface bound antibody is then separately detected from any excess conjugate in the sample by the introduction of an enzyme

30 substrate, such as 4-aminophenol phosphate, from the backside of the membrane which is not gold-plated. The substrate seeps through the membrane and encounters the bound enzyme antibody conjugate at the gold-plated surface. Aminophenol is thus enzymatically generated and detected by oxidation at the gold

35 electrode, the magnitude of the current being a measure of the

concentration of analyte in the sample. However, the sensitivity of the system disclosed in Duan is very low, requiring a 20nA signal compared to 0.1 nA in the present invention. This translates to a 50 times sensitivity advantage when considering actual protein detection limits. The system described by Duan was only capable of detecting protein (human chorionic gonadotropin) down to a level of 500 ng/l, whereas the novel methodology herein described has shown a 10 ng/l protein detection limit.

10 In another experiment reported in Meyerhoff, M. et al., "Novel Nonseparation Sandwich-Type Electrochemical Enzyme Immunoassay System for Detecting Marker Proteins in Undiluted Blood," Clinical Chemistry, 41/9:1378-1384 (1995), a similar microporous membrane was utilized in a non-separation sandwich-type electrochemical enzyme immunoassay system for detecting marker proteins in undiluted blood. However, this method is limited to prostate specific antigen (PSA) measurement in blood. The method described in this reference demonstrates no additional sensitivity when compared to the
20 aforementioned article by Duan. Rather it simply describes the application of the technique to the measurement of an additional protein moiety (prostate specific antigen, PSA).

Niwa, O. et al., "Small-Volume Voltammetric Detection of 4Aminophenol with Interdigitated Array Electrodes and Its
25 Application to Electrochemical Enzyme Immunoassay," Analytical Chemistry, 65:1559-1563 (1993) have reported on the use of an interdigitated array (IDA) micro-electrode cell in small-volume voltammetric detection of 4-aminophenol. However, Niwa used only alkaline phosphatase and used a sensor
30 with a relatively small sensing area measuring 2 x 2 mm and relatively large electrodes of width of 3 to 5 μ m, spaced 2 or 5 μ m from each other. Furthermore, their detection range was from 10 to 1,000 ng/ml for mouse IgG molecules above 1,000 nmol/l for p-aminophenol, which is about 100 times less

sensitive than the present invention and does not make his technique viable for clinical applications with respect to disease-specific antibody detection and quantification.

H. T. Hang et al., in Anal. Him. Acta, 214:187-95 (1988) describes a system for the electrochemical detection of low molecular weight digoxin in the context of an immunoassay, but registers only currents generated by the oxidation of p-aminophenol.

Likewise in the molecular biology field, it is equally important to determine the composition and relative quantities of reactants and products while the reaction is in progress or at its equilibrium endpoint. One specific means of affecting such monitoring utilizes biologic or non-biologic labeling or reporter molecules capable of binding either reactant or product molecules in a structure-restricted manner. Many procedures commonly performed in the field of molecular biology fall into this category. Nucleic acid reactants or products have for many years been directly labeled by a variety of means such as the incorporation of radioactive 32-P or 3-H, or the use of electrophoretic gels incorporating intercalating fluophores such as ethidium bromide. More recently, techniques have been borrowed from the immunochemical or receptor-ligand field and adapted to provide reporter systems that are safer, environmentally friendly, more cost effective, far faster, appropriate for use in a wide range of methods and compatible with efficiently conducting large numbers of procedures. Reporters have recently been introduced into the field of molecular biology that include detection by fluorescence, chemiluminescence, and colorimetry. These labels have been linked or conjugated directly to nucleic acid reactants or products, as well as generated indirectly via nucleic acid-enzyme conjugates in a manner comparable to ELISA techniques. (See, e.g., Tijssen, P., Hybridization With Nucleic Acid Probes: Theory and Nucleic Acid Probes, Vol. 1 (1993); Tijssen, P., Hybridization With

Nucleic Acid Probes: Probe Labeling and Hybridization
Techniques, Vol. 2 (1993); Meier, T. and Fahrenholz, F. eds.,
A Laboratory Guide To Biotin-Labeling in Biomolecule Analysis,
BioMethods Vol. 7 (1996); Garman, A., Non-Radioactive
5 Labelling: A Practical Introduction (Biological Techniques
Series) (1997); Agrawal, S. ed. , Protocols for Oligonucleotide
Conjugates: Synthesis and Analytical Techniques (Methods in
Molecular Biology, Vol. 26) (1993); Burden and Whitney,
Biotechnology: Proteins to PCR: A Course in Strategies and Lab
10 Techniques (1995)) Detection of specific nucleic acid
moieties using such reporters can be effectively performed
while the reaction is in progress (rate measurement or kinetic
measurement), or when the reaction has reached equilibrium
(endpoint reporting). Molecular biology procedures using such
15 reporter systems are commonly applied in many fields such as
biotechnology, environmental protection and public health.

More specifically, recent advances in signal
amplification methods (Dewar R. L. , et al. , "Application of
Branched Chain DNA Signal Amplification to Monitor Human
20 Immunodeficiency Virus Type 1 Burden in Human Plasma," Jrnl of
Inf. Dis. , Vol 170:1172-1179 (1994) as well as template
amplification methods have resulted in a surge of nucleic acid
detection and measurement techniques utilizing enzymatic
conjugates in conjunction with colorimetric or
25 chemiluminescent reporter products in place of the more
hazardous, eco-unfriendly and temporally inefficient
conventional radiographic reporters. All of these new
reporters have, to present, relied on optical detection
methods which, unfortunately, suffer from the infirmities of
30 having requirements for solution clarity, photomultiplication,
complex signal digitalization or analog quantitation and
transmission, viscosity restrictions and requisite background
chromogenic neutrality.

Therefore, despite all of these attempts at improvement,
35 it is still the case that none of these systems describe an

electrochemically detected enzyme-conjugate/reporter substrate capable of providing a cost effective method for direct testing of unprocessed immunochemical and biological samples with a satisfactory level of detection sensitivity.

5 SUMMARY OF THE INVENTION

It is the object of the present invention to provide an immunochemical and molecular biological reporter system to detect and quantify reactants or products. The present invention is intended to include both endpoint and kinetic
10 reporting applications. This system consists of a silicon microchip-formatted interdigitated array (IDA) of closely spaced noble metal electrodes used to detect immunochemical or nucleic acid conjugates containing electrochemically active molecules susceptible to redox recycling and therefore
15 detectable by means of amperimetry or voltammetry.

It is a further object of the present invention to provide a system which can substitute for conventional colorimetric enzyme reporter systems to achieve enhancement relative to performance and economy.

20 It is a further object of the present invention to reduce the time necessary for completion of broad capacity for analytic procedures.

It is a further object of the present invention to increase the detection sensitivity relative to the absolute
25 number of reporter molecules and the volume of solution required for their detection.

It is a further object of the present invention to expand the linear range of concentrations over which reporter molecules may be detected and quantitated.

30 It is a further object of the present invention to broaden the capacity for miniaturization and simplification of equipment relating to both methodologic and detection components supporting both hand-held as well as large-scale high throughput applications.

It is a further object of the present invention to eliminate the sample solution optical clarity or ambient optical density requirements.

It is a further object of the present invention to be
5 able to use microliter or lower specimen requirements which correlates with reduced reagent costs.

It is a further object of the present invention to have manufacturing costs of IDA substantially in comparison to principle components photomultiplier requirements in
10 comparable optical reporter systems.

In one preferred embodiment, this electrochemical reporter system may be applied to an immunochemical method for directing antibodies arising as a result of a viral infection by utilizing an immunoassay including a multivalent enzyme
15 conjugate (Biotin/Avidin) for liberating redox-active molecules, and an IDA for measuring the redox-active molecules. In addition to the first embodiment, this novel reporter system is equally applicable to all enzyme-labeled immunochemistry formats. Methods to which this system applies
20 are commonly, but not exclusively used to examine 1) Infectious diseases (microbial antigen or antibody proteins); 2) Autoimmune diseases (autoantigen or autoantibody proteins); 3) Oncologic markers (so-called tumor specific proteins or steroids); 4) Endocrine hormones (polypeptides, thyronines and
25 steroids); and 5) Therapeutic drugs or toxicologic materials.

In another preferred embodiment, this novel electrochemical reporter system may be applied to detecting or quantifying specific nucleic acids or their amplicons in analytic molecular biologic procedures. Analyses of specific
30 nucleic acids or nucleic acid sequences are gaining wide acceptance and use in the clinical setting to examine body fluids or tissues for the presence of infectious microorganisms, malignancy, inherited disease (genetic defects), forensic medical evidence, and paternity/maternity
35 identification.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features which are considered to be characteristic of the invention are set forth with particularity in the appended claims. The invention itself, however, in respect of its structure, construction, and lay-out, as well as manufacturing techniques, together with other objects and advantages thereof, will be best understood from the ensuing description of preferred embodiments, when read in connection with the appended drawings, in which:

Fig. 1 is a schematic presentation describing a pair of ELISA methods which exemplify the use of optical and electrochemical reporter systems in the immunochemical art.

Figs. 2 and 3 are schematic presentations, including legends, describing the application of the electrochemical reporter system, in accordance with the invention, to differing immunochemical procedural formats. Fig. 2 exemplifies a noncompetitive immunoassay employing a multi-valent labeling conjugate consisting of a biotinylated labeling antibody paired with an avidin-conjugated electrochemical reporter. Fig. 3 exemplifies a competitive immunoassay format in which the labeling antibody (monovalent, and conjugated to the electrochemical reporter) is displaced by an antibody, the analyte, with similar binding specificity.

Fig. 4 is a chart comparing results from six different patients as measured (from left to right in each column) with an indigenous optical system, the electrochemical system in accordance with the invention, and a commercially available assay.

Fig. 5 is a chart which graphically and statistically compares results derived using paired immunoassays which differ in the reporter conjugate employed. An electrochemical reporter in accordance with the invention is used in one immunoassay, and an optical reporter is used in the comparative immunoassay.

Fig. 6 is a chart presenting quantitative HIV RNA results from fourteen human subject plasmas (from left to right in each column) following (1) RT PCR with amplicons quantitated using an electrochemical system in accordance with the invention, and (2) with amplicons quantitated using an optical reporter system.

Figs. 7, 8, and 9 are charts which graphically and statistically compare HIV RNA quantitation results derived following RT PCR and using two matched amplicon detection procedures which differ in the reporter conjugate employed. An electrochemical reporter in accordance with the invention is used in one detection procedure, and an optical reporter is used in the comparative detection procedure.

DETAILED DESCRIPTION OF INVENTION

In its broadest aspect, this electrochemical reporter technology is capable of endpoint detection or kinetic monitoring of clinical and analytical immunochemical and molecular biology procedures including analytical and clinical applications. The present invention employs a closely spaced (nanometer scale) interdigitated array of thin film noble metal microelectrodes to detect voltammetric signals produced in proportion to the concentration of organic (or inorganic) reporter molecules capable of exhibiting redox recycling at the electrode's surface. The anodes and cathodes in the interdigitated array may have a width between about 100 and about 800 nm. Preferred are interdigitated arrays where the widths of the anodes and cathodes is between about 150 and about 650 nm. Most preferred are arrays with anodes and cathodes having a width of about 300 nm. The electrodes may be spaced apart from each other with a distance between about 100 and about 800 nm. Preferred are distances of about 100 to about 650 nm. Most preferred are distances of about 300 nm. The application of the present invention may use ganged IDAs in which a single multipotentiostat is used to detect signals

from several IDAs on a single chip, so that when in use, the read-out(s) show the reaction status of each IDA, as well as combined amperimetric reading(s).

The electrochemical labels may be directly conjugated to the reporter substance, or generated as conjugated or unconjugated products of enzyme/substrate reactions in conjunction with ligand/receptor procedures. Enzyme/substrates which may be used with the present invention include, but are not limited to, α -galactosidase/p-aminophenyl- α -D-galactopyranoside, β -galactosidase/p-aminophenyl- β -D-galactopyranoside, α -glucosidase/p-aminophenyl- α -D-glucopyranoside, β -glucosidase/p-aminophenyl- β -D-glucopyranoside, α -mannosidase/p-aminophenyl- α -D-mannopyranoside, β -mannosidase/p-aminophenyl- β -D-mannopyranoside, acid phosphatase/p-aminophenylphosphate, alkaline phosphatase/p-aminophenylphosphate, and phosphodiesterase II/p-aminophenylphosphorylcholine.

Immunochemistry

Immunoassays for detecting an indicative species have been adapted to numerous procedural formats. For example, relevant reactions may all take place in solution, or specific components may be anchored to solid supports for ready separation of bound ligands. Further examples of format variety include the use of labeled recognition molecules to directly indicate the presence of a substance of interest (non-competition assay), or specific recognition molecules, in selectively limited quantities, may be reacted with a sample containing the endogenous substance of interest as well as a known quantity of an exogenous label, with similar binding characteristics as the substance of interest, but conjugated with a detectable label. In such a format (competition assay), the quantity of labeled material complexed to the recognition molecules will be dependent on the relative ratios of the endogenous (sample) and exogenous (label) molecules.

The conjugate is not necessarily restricted to the molecular species eventually subjected to detection. In the preferred immunoassay example, the EIA or ELISA, protein analytes contained in a given sample as antibodies are, for instance, bound to an antigen or capture antibody. Subsequently, the sample matrix is washed off, and the analyte is bound in a quantitative relationship to an enzyme label after another washing step to remove the excess enzyme-labeled antibodies. If colorimetric detection is to be utilized, the quantity of enzyme attached to the solid phase may be determined by adding a specific substrate and measuring the amount or rate of enzymatically generated colored product. This endpoint quantity or rate is a proportional measure of the amount of antigen present in the specimen.

The electrochemical reporter system can be generally substituted in place of conventional colorimetric or chemiluminescent enzyme-linked immunoassay reporters and applied throughout the existing range of analytical test formats. These methodologies are commonly, but not exclusively, used to examine blood or other body fluids for the presence of substances associated with: 1) Infectious diseases (microbial antigen or antibody proteins); 2) Autoimmune diseases (autoantigen or autoantibody proteins); 3) Oncologic markers (so-called tumor specific proteins or steroids); 4) Endocrine hormones (polypeptides, thyronines and steroids); and 5) Therapeutic drugs or toxicologic materials.

In the first embodiment, the invention is used to detect antigens or antibodies. The term "antibody" refers to immunoglobulins of any isotype or subclass as well as any fab or fe fragment of the aforementioned. Antibodies of any source are applicable including polyclonal materials obtained from any animal species; monoclonal antibodies from any hybridoma source; and all immunoglobulins (or fragments) generated using viral, prokaryotic or eukaryotic expression systems. Biologic recognition molecules other than

antibodies, are equally applicable for use with the current invention. These include, but are not limited to: cell adhesion molecules, cell surface receptor molecules, and solubilized binding proteins. Non-biologic binding molecules, such as "molecular imprints" (synthetic polymers with pre-determined specifically for binding/complex formation), are also applicable to the invention. The terms "antigens," "immunogens" or "haptens" refer to substances which can be recognized by in vivo or in vitro immune elements, and are capable of eliciting a cellular or humoral immunologic response. Although the electrochemically active reporter utilized in the embodiment is specified as para-aminophenol (generated by the action of a beta-galactosidase conjugate in conjunction with a specific substrate), it should be noted that the invention is generally applicable to molecules capable of redox recycling, and enzyme systems capable of generating such reporters.

In particular, the embodiment will be described in the context of electrochemical detecting antibody to the human immunodeficiency virus (HIV). More specifically, the detection of antibodies developed in vivo as a result of infection with the human immunodeficiency virus.

An electrochemical sensor comprised of an array of interdigitated micro electrodes of the kind useful in the practice of the present invention is published in International Patent Application having PCT Publication No. WO 94/29708 (published Dec. 22, 1994). The application discloses an array consisting of four pairs of comb-shaped interdigitated anodes and cathodes serially arranged on a planar silicon chip. Particular circumstances may, of course, require that more or fewer electrodes are mounted in an array. For instance, planar counter and reference electrodes of the type commonly used in electrochemical cells may be arranged besides the interdigitated electrodes. Conductors connecting

the electrodes to electrical contact surfaces are covered by an insulating layer.

Referring now to FIG. 1, a schematic presentation describing a pair of enzyme-linked immunoassays which exemplify and compare the use of optical and electrochemical reporter systems is shown.

As shown in FIG. 1, each member of the paired immunoassays is first coated with HIV p24 as an antigenic substrate. Each of the immunoassay pairs is then incubated with serum from HIV positive volunteers which contain antibodies to p24, thereby affording complexation of anti p24 antibodies with a target antigen substrate. Each member of the paired immunoassays is then incubated with goat anti-human immunoglobulin conjugated to biotin. At this point, one of the paired immunoassays is treated with 1) avidin/horseradish peroxidase conjugate and the other paired immunoassays is treated with beta galactosidase/avidin conjugate producing matched immunoassays with optical and electrochemical reporters respectively.

Referring now to FIG. 3, an enzyme-linked immunoassay and electrochemical detection in accordance with the invention is shown. In the first step, unlabeled antigen is bound to a solid phase matrix in a well-known manner. The solid phase matrix may be glassy or polymeric beads, micro titer plates, porous or impervious or even fibrous matrices or membranes or the like, as is well-known in the art. Binding of the antigen or protein to the matrix may be accomplished non-specifically by absorption or covalently by any of the well-known chemical coupling methods. The antigen may be a specific composition of any of the HIV proteins, either recombinant or isolated, or a viral lysate or peptide, or a specific composition of HIV peptides and proteins.

The second step involves attaching antibody having one or more enzyme labels, such as β -galactosidase or alkaline phosphatase either covalently or by an affinity bond (ionic,

hydrogen or hydrophobic, as the case may be) until maximum saturation of the binding site has been reached. The antibodies may be HIV-antibodies, either whole and/or different fragments thereof, with or without hybrid epitope recognition sites, recombinantly expressed, or viral libraries or from immunized animals. They may be of a single class of antibody or a defined mixture of antibodies of different classes with comparable or specific antigen affinities.

The third step requires a sample of body fluid containing the analyte such as HIV antibodies of the kind referred to above to be added. Following an appropriate incubation period and washing steps, a substrate such as p-aminophenyl-g-galactopyranoside is added. Where β -galactosidase is used as the enzyme label of the antibody, the resulting product is p-aminophenol.

The quantity of p-aminophenol produced is indicative of the concentration of antibody in the specimen and can be measured using an array of interdigitated micro electrodes when the p-aminophenol reacts at the interdigitated anodes and cathodes thereof in a redox process repeatedly alternating between the p-aminophenol and quinoneimine.

Preferably, when the p-aminophenol reacts at the interdigitated anode and cathode of the microelectrode, a redox reaction repeatedly generated between p-aminophenol and the corresponding quinone. Detection or measurement of current may then be accomplished by following an endpoint or kinetic amperimetry. In other words, the current generated by the redox reaction may be measured after a certain time following commencement of the redox operation, or it may be performed dynamically by measuring the rise in current.

Referring now to FIG. 2, a capture immunoassay including the application of multiple bonded enzymes of biotin-avidin conjugates as a method of further signal amplification in accordance with the invention is shown. Initially, antigen, purified HIV p24, is bound to a solid phase carrier matrix

Immulon II 96-well Microtiter plates (Dynal Corporation). Subsequently, plasma from uninfected volunteers or HIV seropositive patients containing α -p24 antibodies, are added to complex with the p24 antigens bound to the solid matrix.

- 5 In the next step, an affinity ligand conjugate is added and allowed to bind before a polyvalent affinity receptor labeling conjugate is introduced. Finally, an enzyme substrate, for example, p-aminophenol- β -galactopyranoside is allowed to react with the labeling enzymes to produce a large quantity of
- 10 p-aminophenol which when subjected to redox recycling on the interdigitated array of anodes and cathodes of a microelectronic sensor will recycle between p-aminophenol and quinoneimine, thus delivering a strong electrical signal indicative of the concentration of antibody present in the
- 15 specimen.

It will be appreciated by those skilled in the art that the immunochemical aspects of the above embodiments require certain preparatory steps which included, at least, the following:

- 20 The antigen was bound to the solid phase matrix in a glycine buffer coating solution prepared by adding reconstituted p24 (Intracel, Inc.) to the coating buffer solution to a concentration of 5 μ g/ml of p24.

- 25 Washing buffer solutions were applied consisting of Dulbecco's phosphate buffered saline solution of pH 7.4 (DPBS free of calcium and magnesium) obtained from BioWhittaker and containing 137 mM of sodium chloride, 3 mM of potassium chloride, a mM of sodium hydrogen phosphate and 1.5 mM of potassium dihydrogen phosphate.

- 30 Antibody was diluted in a buffer consisting of DPBS with 1% (weight/volume) of bovine serum albumin, heat shock quality (Jackson) and 0.01% (w/v) Tween 20 (10% w/v) (Boehringer).

Blocking buffer and solution was prepared from DPBS with 5% (w/v) of bovine serum albumin as above and 0.01% (w/v) of Tween 20 (10% w/v) (Boehringer).

The enzyme substrate buffer was prepared from 100 mM of sodium chloride in 100mM of sodium phosphate buffer at pH 7.25. The enzyme substrate solution was made by dissolving p-aminophenol- β -D-galactopyranoside (Sigma) at a molarity of 1.5 mM in the enzyme substrate buffer.

Referring now to Fig. 4, a schematic presentation of the invention as applied to the detection of p24 antibody in serum is shown. Preferably, recombinant p24 was non-unspecifically bound by absorption to wells of highly adsorptive micro titer plates by incubating 50 μ l of the above coating solution for 2 hours at 37° Celsius. Alternately, microtiter plate coating could be accomplished by overnight incubation at 4° Celsius. The wells were then washed twice with 150 μ l of the above washing buffer at room temperature and thereafter blocked with 100 μ l blocking solution for 2 hours at room temperature. During this time the samples were repeatedly shaken.

Thereafter, the wells were again washed three times at room temperature, with 150 μ l of the patients' serum diluted with dilution buffer at factors ranging between 1:30 and 1:30,000 added and incubated at 37° Celsius for 1 hour. The wells were then washed three times with 150 μ l washing buffer at room temperature. 50 μ l of a solution containing biotinylated Fc-Fab₂ antibody fragments (Jackson) diluted 1:10,000 in dilution buffer were added to each well to detect specifically bound p24 antibody and incubated at 37° Celsius for 1 hour. The wells were then again washed three times with 200 μ l of washing buffer at room temperature. 50 μ l of avidinD- β -galactosidase conjugate (Vector). C-10 μ g/ml diluted in dilution buffer was added and incubated for .5 hour at room temperature to detect specifically bound biotinylated Fc-Fab₂' antibody fragments. After washing the wells three

times with 150 μ l of washing buffer at room temperature, 170 μ l of the enzyme substrate solution was added to the wells.

After incubation for about 30 minutes to free the electrochemically redox active p-aminophenol from the p-aminophenol- β -D-galactosidase, the respective supernatants were individually aspirated and transferred to 1 ml sealable plastic vials. To neutralize any remaining residual enzyme activity the vials were incubated at 80° Celsius in a water bath for 10 minutes and were cooled in ice water to room temperature. For a maximum neutralization or inactivation of the activity of the avidin D- β -galactosidase, the inactivation temperature was determined for the bound avidin D- β -galactopyranoside.

The inactive or neutralized supernatant was then transferred to a flow chamber at the bottom of which the microsensor was positioned so as to determine any redox current resulting from the recycling of the enzymatically freed p-aminophenol. Relative to a silver/silver chloride reference electrode, a potential of +250 mV was applied to the anodes of the interdigitated thin-film metal electrodes and a potential of -50 mV is applied to the cathodes. The measurable anode and cathode currents were found to correspond to the presence and quantity of specifically bound antibody to p24.

The redox current clearly distinguished between positive and negative blood samples, and in the positive blood samples it proportionally reflected differences in concentration of p24 antibodies in the serum specimens. In all instances, the electrochemically measured signals corresponded to results obtained by optical read-outs of the same samples. For control purposes, endpoint titers obtained by optical state of the art enzyme-linked immunosorbent assays as disclosed in Abbot Laboratories' Enzyme Immunoassay for the Detection and Semiquantitation of Antibody to the p24 (core) Protein of the

Human Immunodeficiency Virus Type 1 (HIV-1) in Human Serum or Plasma (hereinafter "Abbot Immunoassay") and by the method in accordance with the invention were compared with each other and were found to differ insignificantly.

5 Referring now to FIG. 4, a chart comparing the results of measuring endpoint titers performed on samples obtained from six different HIV seropositive patients as measured (from left to right in each column) with an indigenous optical system, the electrochemical system in accordance with the invention,
10 and a commercially available assay, is shown. One measurement (the left column in the figure) was performed with optical read-out by an indigenously developed reference or comparative p24 immunoassay. Another measurement (the right column in the figure) was performed with a commercial Abbott immunoassay,
15 and one measurement (the middle column in the figure) was taken electrochemically by the method in accordance with the invention. The results of the three measurements will be seen to be substantially identical for each patient. The endpoint titer reflects the dilution factor of the serum where the
20 detectable signal obtained with the HIV positive serum is equal to the mean plus or minus two times the standard deviation relative to signals generated when multiple HIV negative serum samples are similarly analyzed. Paired results and regression analyses are presented in Fig. 5. Both
25 correlation coefficient (R squared) and the slop of the least squares best fit line approach 1.0, attesting the comparability of results derived by the optical and electrochemical reporter methods.

Molecular Biology

30 The present invention is also capable of endpoint detection or kinetic monitoring molecular biology procedures including analytical and clinical applications. The same interdigitated arrays mentioned above can be used to detect voltammetric signals produced in proportion to the
35 concentration of organic (or inorganic) reporter molecules

capable of exhibiting redox recycling at the electrode's surface.

These electrochemical labels may be (1) produced as nucleic acid amplicon conjugates which are generated during the replication of selected templates in conjunction with procedures which are exemplified, but not necessarily limited to, PCR (polymerase chain reaction), LCR (ligase chain reaction), NASBA (nucleic acid sequence based amplification), SDA (strand displacement amplification), TAS (transcription based amplification system), 3SR (self-sustained sequence replication) and Q-beta replicase systems; (2) used as substrate moieties or directly conjugated to the complex multiple termini of poly-branched nucleotide targeting probes utilized in signal amplification methods for the detection of specific nucleotide sequences (e.g.: branched Chain DNA technology); or (3) the electrochemically reactive reporters or associated enzymes labels may be directly conjugated to specified nucleic acid reactants or products.

In a molecular biology embodiment, an enzyme mediated electrochemical reporter system, utilizing the IDA previously described, was applied to an analytical method for the detection and quantitation of nucleic acid fragments. In particular, the example used to illustrate the system's general applicability in this field compared the use of the novel electrochemical reporter system to a commercially available and FDA approved polymerase chain reaction (PCR) methodology for the quantitation of HIV RNA in human plasma.

A pair of HIV Monitor' kits produced by Roche Molecular Systems (Branchburg, NJ) were each applied per instructions contained in the package insert, to a series of five plasmas from HIV seronegative volunteers and nine plasmas obtained from HIV patients. For one kit, all preparation, amplification and detection systems were processed entirely per the instructions contained in the package insert. Detection of the resulting PCR product, however, was

accomplished using an avidinated - beta galactosidase conjugate (10Mg/ml avidin D- β -galactosidase, Vector Labs, Burlingame, CA) dissolved in phosphate buffered saline (pH 7.4) PBS with 1% BSA and 0.01% Tween 20 which bound in solution to the biotinylated amplicons. The labeled nucleic acid-enzyme complexes were then separated from solution by hybridization to capture oligomers anchored to the microtiter plate-wells (RMS monitor™).

Following incubation with substrate, β -galactopyranoside (60 mM p-aminophenol- β -D-galactopyranoside, Sigma Chemical, St. Louis, MO) dissolved in PBS, 7.4), the resulting electrochemically active product (PAP) was measured amperometrically utilizing the interdigitated micro electrode array. Paired results are presented in Fig. 6 and regression analyses are presented in FIGS. 7-9. Both the correlation coefficient (R squared) and the slope of the least squares best fit line approach 1.0, attesting to the comparability of results derived by the optical and electrochemical reporter methods.

Side by side analyses of HIV-1 negative and HIV-1 positive human plasma samples were carried out using the RMS Monitor procedure per the package insert as well as utilizing the electrochemical reporter modification. Comparison of the results demonstrated the analytic comparability of the two methods. The electromechanical method, is equally applicable as a simple modification to all currently available enzymatically mediated or direct optical reporter methods, and in a manner similar to aforementioned immunoassay applications, substantially simplifies the detector requirements attending conventional calorimetric or chemiluminescent conjugates.

Such methods are currently used commercially for assaying a wide range of specific microbe/virologic and genetic RNA and DNA sequences. In each case, these methods are readily

amenable to the minor chemical modification necessary to afford general substitution by electrochemical reporter detection and quantitation using the interdigitated micro electrode array as previously described.

5 It will be appreciated by those skilled in the art that, the present invention which utilizes thin-film microelectrode arrays and redox recyclable reporter molecules is also applicable, in general, to the examination of tissue, blood or other body fluids for the presence of nucleic acids associated
10 with: 1) infectious diseases; 2) autoimmune diseases; 3) malignancy; 4) inherited diseases; 5) maternity/paternity identification. It will be equally well appreciated by those skilled in the art that the present invention can also be used in both basic research and applied science procedures
15 conducted outside the clinical setting in a broad range of disciplines exemplified, but not limited to: 1) forensic science; 2) basic cellular and developmental biology; 3) archeology and paleontology; and 4) the wide range of animal and plant biotechnologies employing recombinant DNA
20 procedures.

It will be appreciated by those skilled in the art that, the present invention which utilizes thin-film microelectrode arrays and redox recyclable reporter molecules is also applicable to the examination of blood, body fluids, or
25 tissues for the purpose of detecting and measuring the following: 1) free or complex immunoglobulins associated with health or disease (infection or autoimmunity); 2) the presence of antigens related to normal and abnormal developmental processes, (infectious or autoimmunity); 3) presence or
30 absence of specific nucleotide sequences associated with normal or abnormal genetic development, malignancy, or infectious disease.

Having described and illustrated the principles of our invention with reference to a preferred embodiment, it will be
35 apparent that the invention can be modified in arrangement and

detail without departing from such principles. As such, it should be recognized that the detailed embodiment is illustrative only and should not be taken as limiting the scope of our invention. Rather, we claim as our invention all
5 such embodiments as may fall within the scope and spirit of the following claims and equivalents thereto.

We claim:

1. An electrochemical reporter system comprising:
 - 5 (a) a recognition molecule capable of specifically binding an analyte in a structure restricted manner;
 - (b) an enzyme;
 - (c) a coupling element, for coupling with specificity the enzyme to the recognition molecule or the analyte;
 - 10 (d) a substrate which in the presence of the enzyme is cleavable into a reporter molecule capable of exhibiting redox recycling; and
 - (e) a sensor for detecting the electrochemical reporter molecule, said sensor having a configuration such
15 that the reporter molecule will exhibit redox recycling.
2. The electrochemical reporter system of claim 1, the sensor being a microelectronic interdigitated array of
20 electrodes with a distance between the electrodes of about 100 to about 800 nanometers.
3. The electrochemical reporter system of claim 2, the sensor being a microelectronic interdigitated array of
25 electrodes having a distance between the electrodes of about 300 nanometers.
4. The electrochemical reporter system of claim 1, the recognition molecule being selected from the group consisting
30 of immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, and non-biologic binding molecules.

5. The electrochemical reporter system of claim 1, the enzyme being capable of effecting the cleavage of a covalent bond of the substrate.

5 6. The electrochemical reporter system of claim 5, the enzyme being selected from the group consisting of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase, β -mannosidase, acid phosphatase, alkaline phosphatase and phosphodiesterase II.

10

7. The electrochemical reporter system of claim 1, the substrate being selected from the group consisting of p-aminophenyl- β -D-galactopyranoside, p-aminophenyl- α -D-galactopyranoside, p-aminophenyl- α -D-glucopyranoside, p-aminophenyl- β -D-glucopyranoside, p-aminophenyl- α -D-mannopyranoside, p-aminophenyl- β -D-mannopyranoside, p-aminophenylphosphate, and p-aminophenylphosphorylcholine.

8. The electrochemical reporter system of claim 1 wherein
20 the coupling element is selected from the group consisting of:

- (a) biotin conjugated to an antibody specific for the recognition molecule or the analyte and avidin conjugated to the enzyme;
- 25 (b) biotin conjugated to an antibody specific for the recognition molecule or the analyte and streptavidin conjugated to the enzyme;
- (c) digoxigenin conjugated to an antibody specific for the recognition molecule or the analyte and digoxigenin-specific antibody conjugated enzyme
- 30 (d) by cross-linking the enzyme to the recognition molecule; and
- (e) by cross-linking the enzyme to an antibody specific for the recognition molecule.

9. The electrochemical reporter system of claim 1 wherein the substrate is cleaved into at least one component comprising para-aminophenol.

5 10. The electrochemical reporter system of claim 1 wherein the sensor is a microelectronic interdigitated array of electrodes having width between about 100 and about 800 nanometers and spaced between about 100 and about 800 nanometers from each other.

10

11. An electrochemical immunoassay for detecting an analyte in a sample comprising the steps of:

- 15 (a) having linked to a surface an antigen with an antibody specific for an analyte bound to the antigen, the antibody being coupled to an enzyme or having a coupling element for being specifically coupled to an enzyme;
- (b) contacting the surface with a sample to be analyzed;
- 20 (c) collecting the antibodies displaced from the antigen by analyte in the sample;
- (d) adding a substrate to the collected antibodies, said substrate being cleavable by the enzyme into an electrochemical component; and
- 25 (e) measuring the presence or amount of electrochemical with an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.

12. An electrochemical immunoassay for detecting an analyte in a sample comprising the steps of:

- 30 (a) having linked to a surface an antibody specific for an analyte and an antigen bound to the antibody, the antigen being coupled to an enzyme or having a means of being specifically coupled to an enzyme;
- (b) contacting the surface with a sample to be analyzed;

- (c) collecting the antigen displaced from the antibody by analyte in the sample;
- (d) adding a substrate to the collected antigen, said substrate being cleavable by the enzyme into an electrochemical component; and
- 5 (e) measuring the presence or amount of electrochemical with an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.
- 10 13. An electrochemical immunoassay for detecting a specific analyte in a sample comprising the steps of:
- (a) having a recognition molecule linked to a surface, said recognition molecule capable of specifically binding the analyte in a structure restricted
- 15 manner;
- (b) contacting the surface with a sample to be analyzed;
- (c) coupling with specificity an enzyme to the recognition molecule or the analyte;
- (d) adding a substrate, which in the presence of the enzyme is cleaved into a reporter molecule capable
- 20 of exhibiting redox recycling; and
- (e) measuring the presence or amount of electrochemical with an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.
- 25 14. The electrochemical immunoassay of claim 13 wherein the enzyme is coupled with specificity to the recognition molecule or the analyte by having the enzyme be avidin- or streptavidin- conjugated and a biotin labeled antibody
- 30 specific to the recognition molecule or analyte.
15. An electrochemical reporter system comprising:
- a) an enzyme;

- b) a coupling element, for coupling the enzyme to a nucleotide, oligonucleotide, branched oligonucleotide, nucleic acid, or analogs thereof;
- c) a substrate, which in the presence of the enzyme is
5 cleavable into a reporter molecule capable of exhibiting redox recycling; and
- d) a sensor for detecting the reporter molecule, the sensor having a configuration such that the reporter molecule exhibits redox recycling.
- 10
16. The electrochemical reporter system of claim 15, the sensor being a microelectronic interdigitated array of electrodes with a distance between the electrodes of between about 100 to about 800 nanometers.
- 15
17. The electrochemical reporter system of claim 16, the distance between the electrodes being about 300 nanometers.
18. The electrochemical reporter system of claim 15, the
20 enzyme being capable of effecting the cleavage of a covalent bond of the substrate.
19. The electrochemical reporter system of claim 18, the enzyme being selected from the group consisting of α -
25 galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase, β -mannosidase, acid phosphatase, alkaline phosphatase and phosphodiesterase II.
20. The electrochemical reporter system of claim 15, the
30 substrate being selected from the group consisting of p-aminophenyl- β -D-galactopyranoside, p-aminophenyl- α -D-galactopyranoside, p-aminophenyl- α -D-glucopyranoside, p-aminophenyl- β -D-glucopyranoside, p-aminophenyl- α -D-

mannopyranoside, p-aminophenyl- β -D-mannopyranoside, p-aminophenylphosphate, and p-aminophenylphosphorylcholine.

21. The electrochemical reporter system of claim 15, the
5 coupling element being selected from the group consisting of
a) a covalent bond;
b) biotinilated nucleotide, oligonucleotide, branched
oligonucleotide, nucleic acid, or analogs thereof and avidin
or streptavidin conjugated enzyme; and
10 c) digoxigenin labeled nucleotide, oligonucleotide, branched
oligonucleotide, nucleic acid, or analogs thereof and
digoxigenin-specific antibody conjugated enzyme.

22. The electrochemical reporter system of claim 15, the
15 substrate being cleaved into at least one component comprising
para-aminophenol.

23. The electrochemical reporter system of claim 15, the
sensor being a microelectronic interdigitated array of
20 electrodes having width between about 100 and about 800
nanometers and spaced between about 100 and about 800
nanometers from each other.

24. An assay for detecting or quantitating a specific nucleic
25 acid sequence in a sample comprising the steps of:
a) having a first single stranded nucleic acid having a
sequence complementary to a first segment of the specific
nucleic acid sequence to be detected or quantified, the
first single stranded nucleic acid being in a solution and
30 bound to a surface;
b) contacting the first single stranded nucleic acid with a
sample to be analyzed;
c) adding a labeled second single stranded nucleic acid to the
sample to be analyzed, said second nucleic acid having a

sequence complementary to a second segment of the nucleic acid sequence to be detected or quantified;

d) adding a substrate, said substrate in the presence of the second single stranded nucleic acid being cleaved into an electrochemical; and

e) detecting the presence or measuring the amount of electrochemical present in the solution with an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.

10

25. An electrochemical assay for detecting or quantitating a specific nucleic acid sequence in a sample comprising the steps of:

a) amplifying the specific nucleic acid sequence in the

15 presence of labeled nucleotides or labeled primer oligonucleotides such that the labeled nucleotides or the labeled primer oligonucleotides are incorporated in labeled newly synthesized nucleic acid;

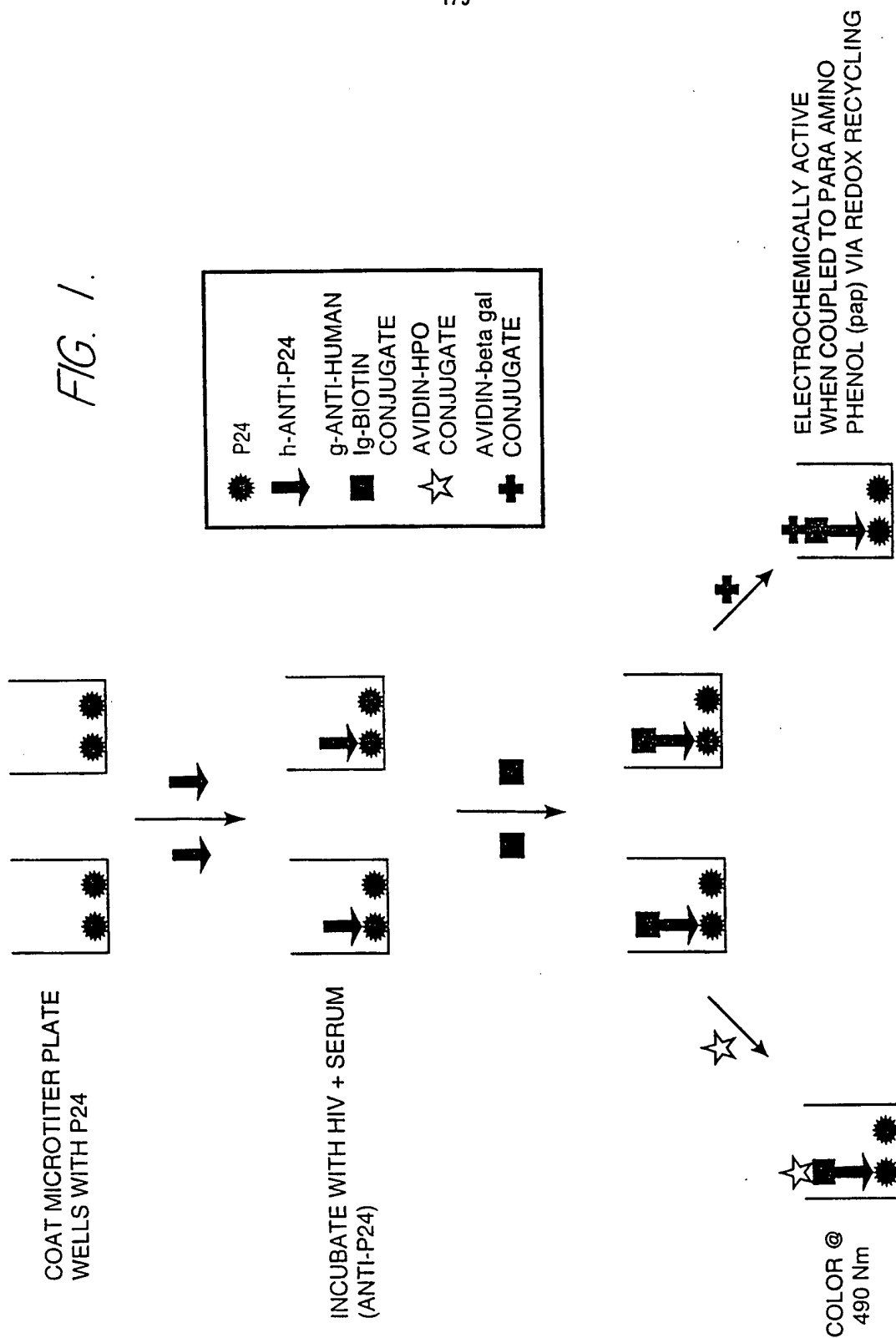
b) adding a substrate, said substrate in the presence of the

20 labeled newly synthesized nucleic acid being cleaved into an electrochemical; and

c) measuring the presence or amount of the electrochemical present with an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.

25

FIG. 1.



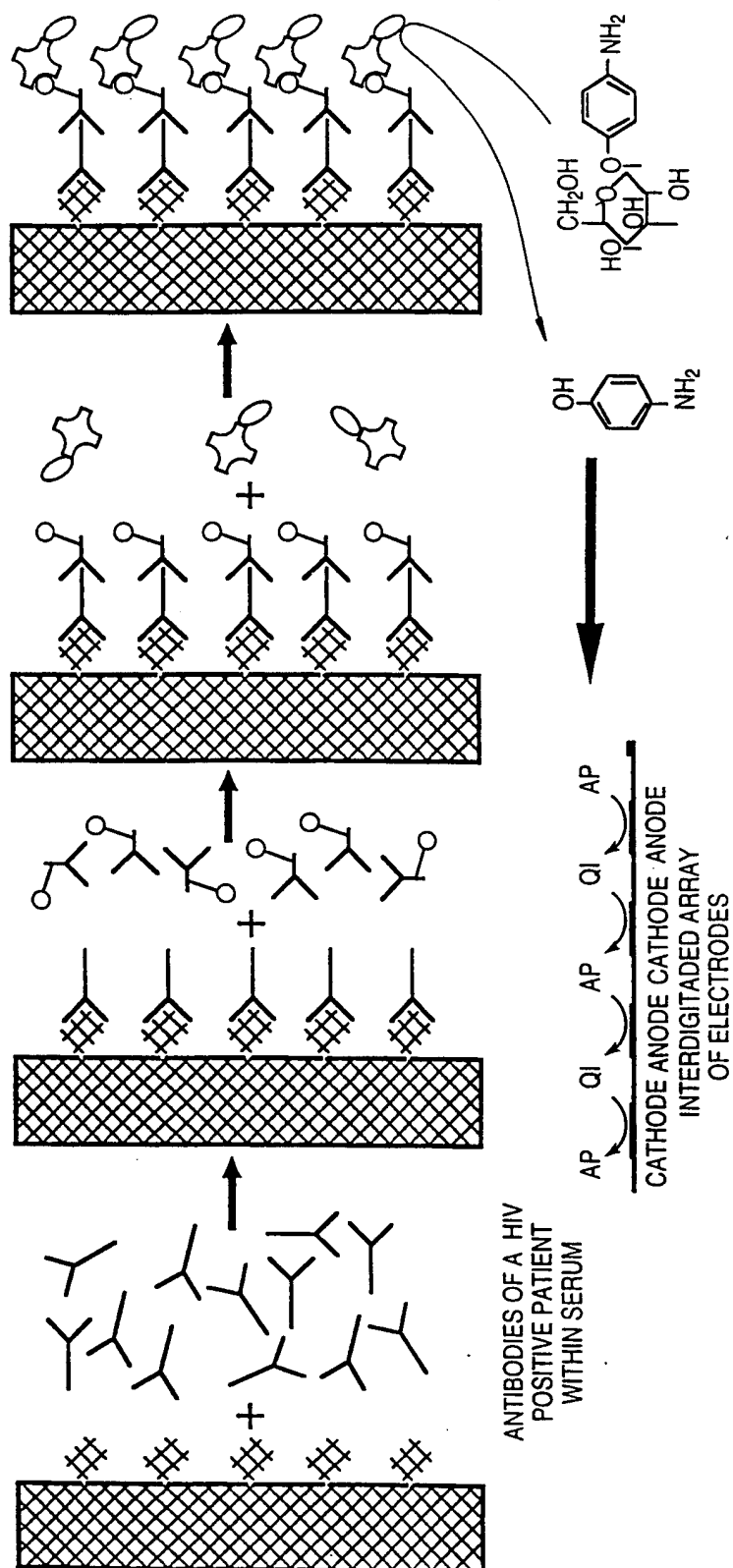








FIG. 2.

	MICROTITER PLATE		p24		HUMAN <p24>-IgG (SERUM)	AP	p-AMINOPHENOL
	<Fc>Fab2'-BIOTIN		AVIDIN LABELED WITH β -GALACTOSIDASE		QUINONEIMINE	QI	

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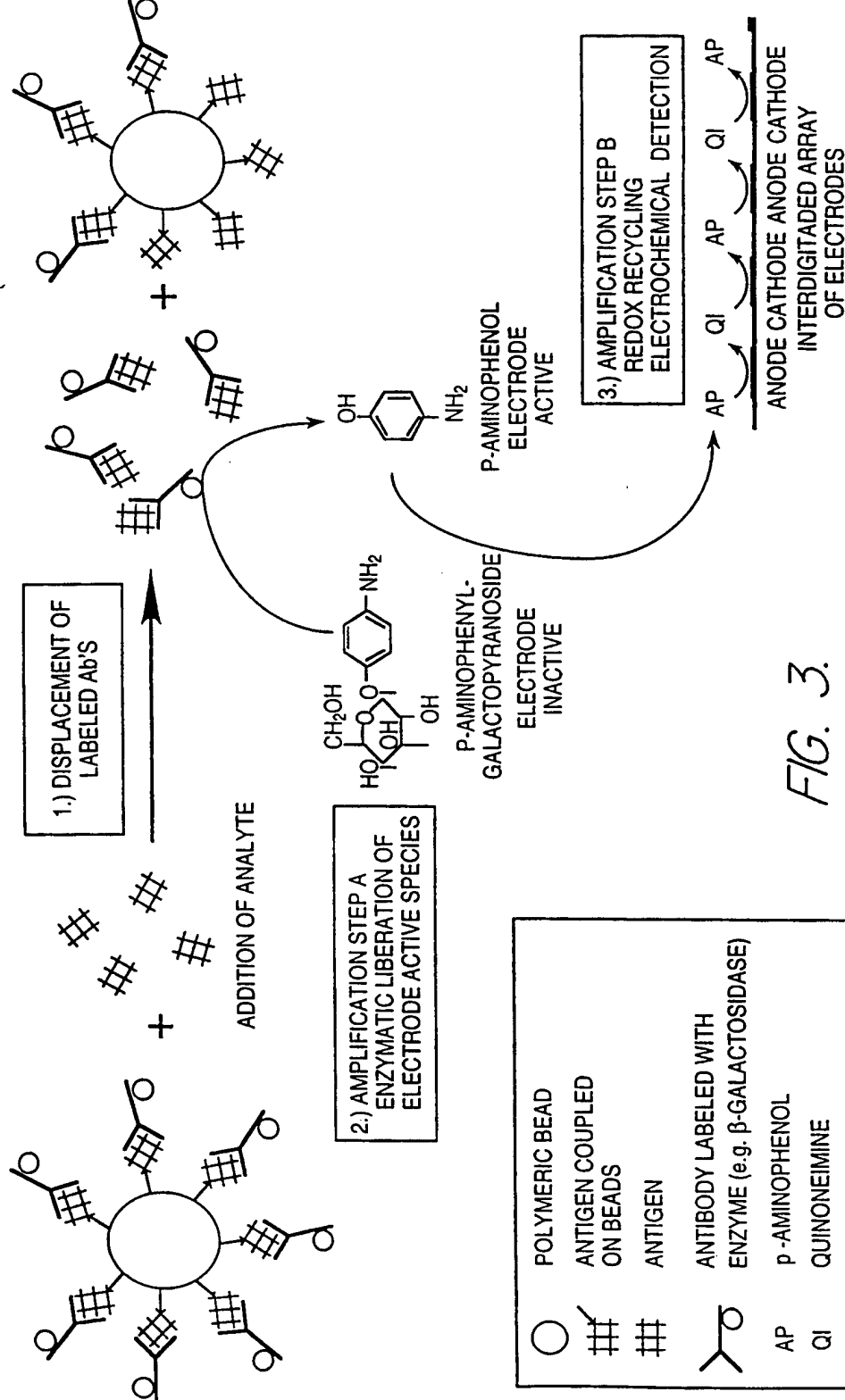
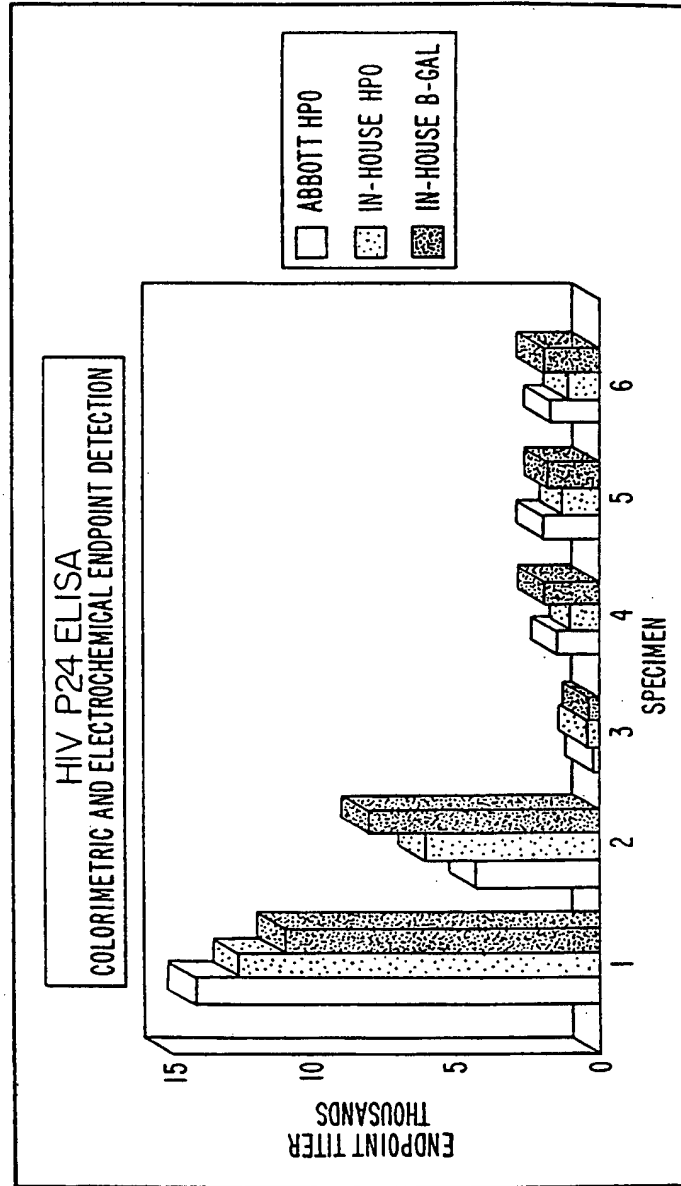


FIG. 3.

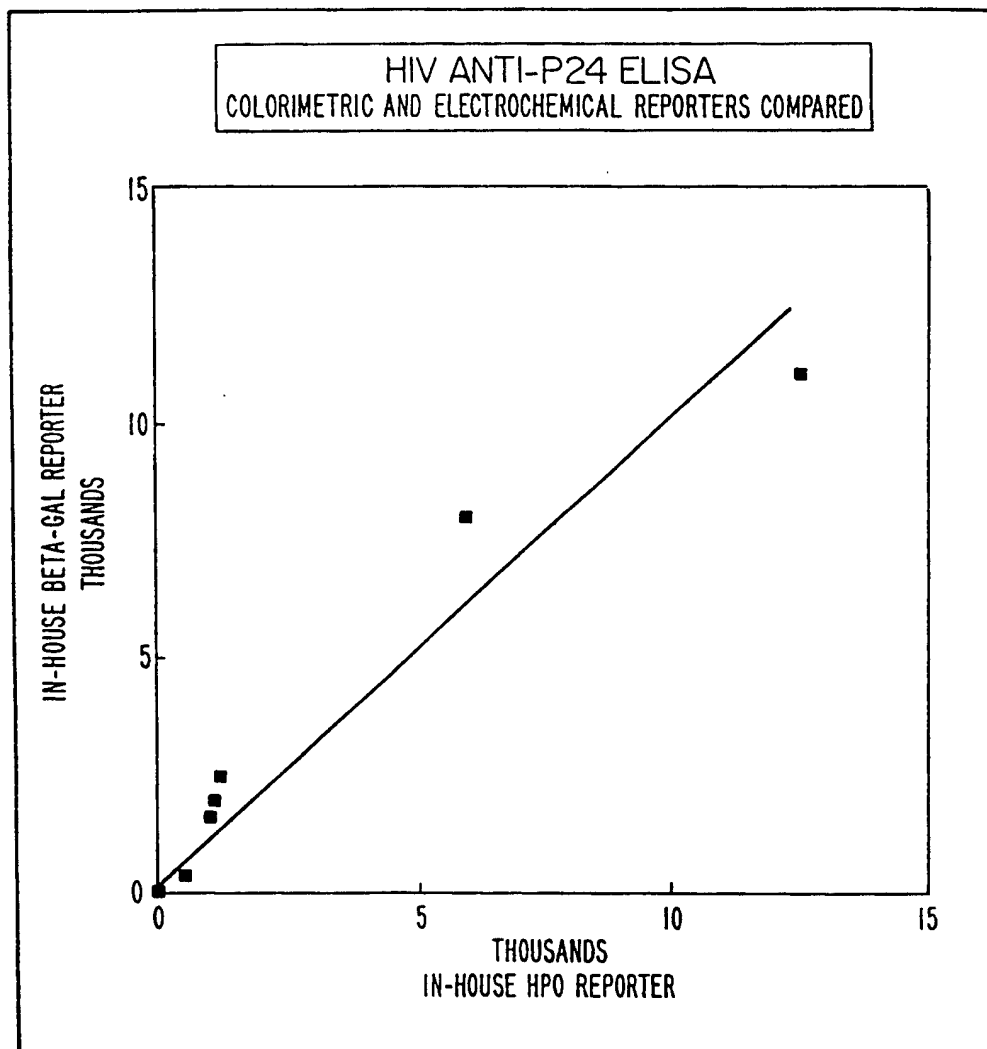
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1	14000	12500	11000
2	4400	6000	8000
3	300	500	450
4	1500	1000	2500
5	2000	1200	1700
6	1700	1100	2000
7	NEG	NEG	NEG
8	NEG	NEG	NEG
9	NEG	NEG	NEG
10	NEG	NEG	NEG

FIG. 4.



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FIG. 5.



REGRESSION OUTPUT:

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STD ERR OF Y EST	1003.611
R SQUARED	0.944968
NO. OF OBSERVATIONS	10
DEGREES OF FREEDOM	8

X COEFFICIENT(S)	1.021692
STD ERR OF COEF.	0.087172

SUBSTITUTE SHEET (RULE 26)

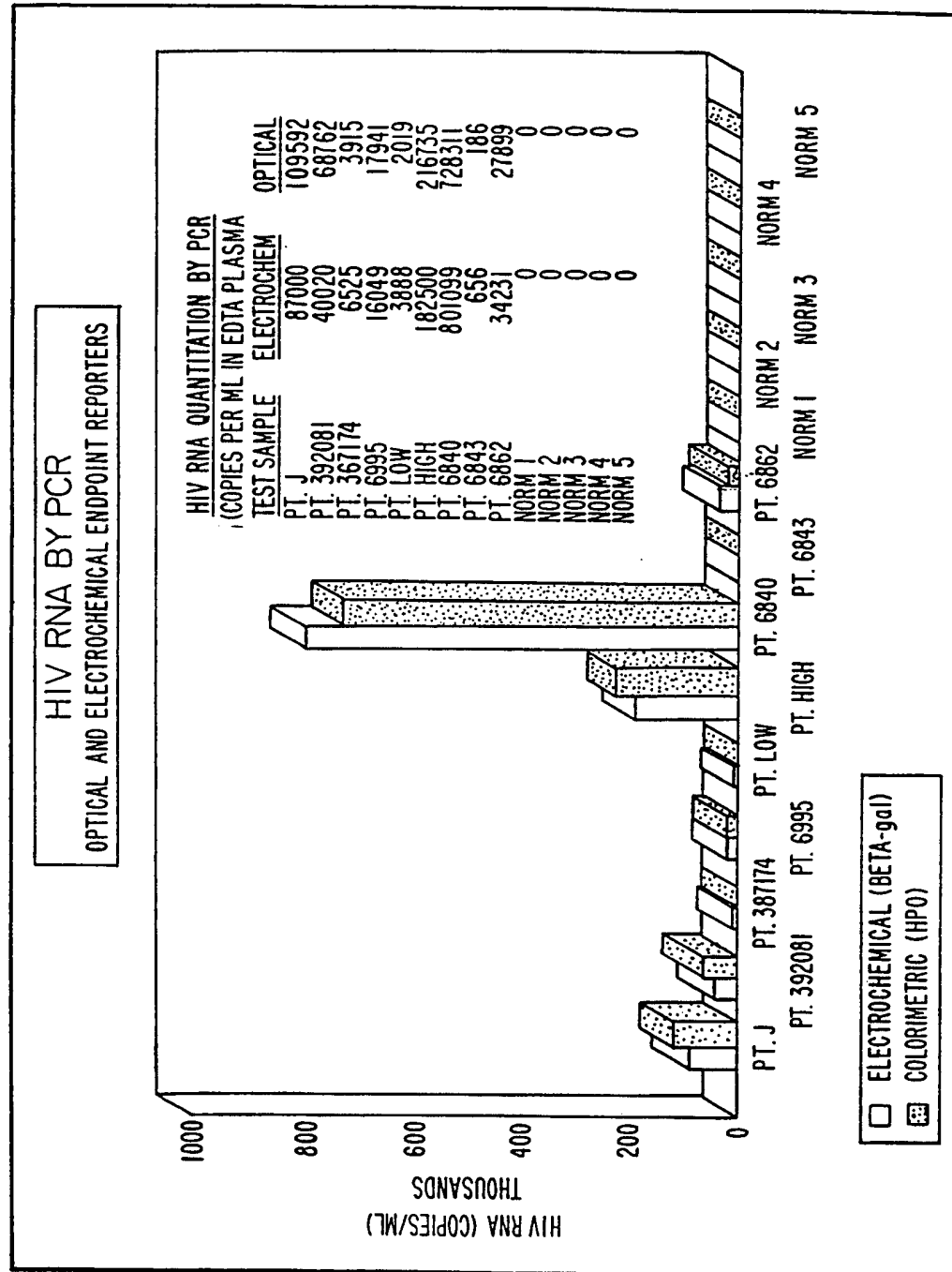
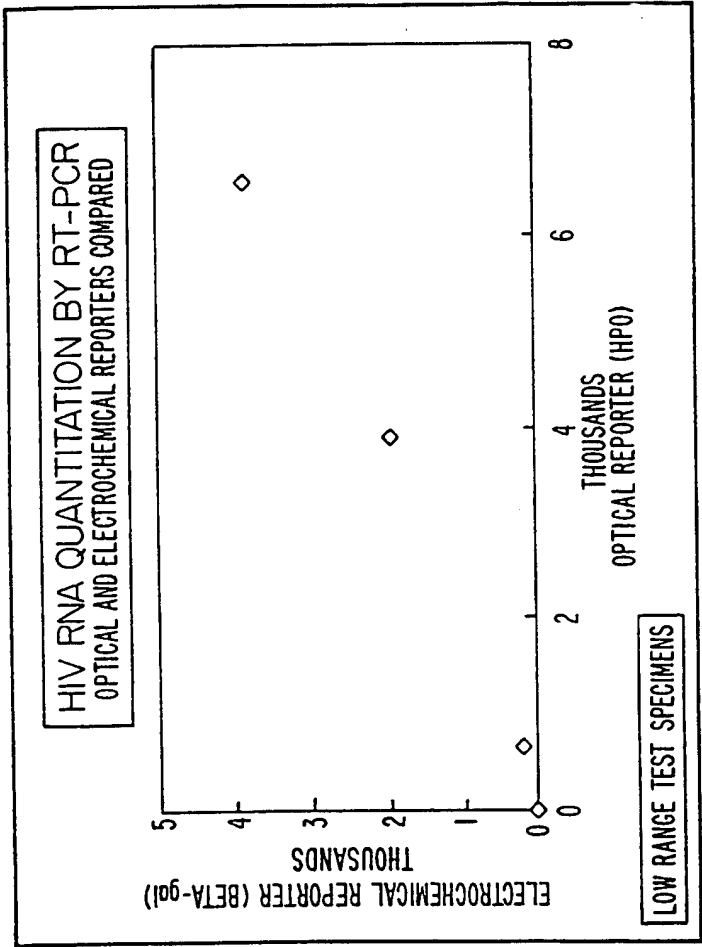


FIG. 6.

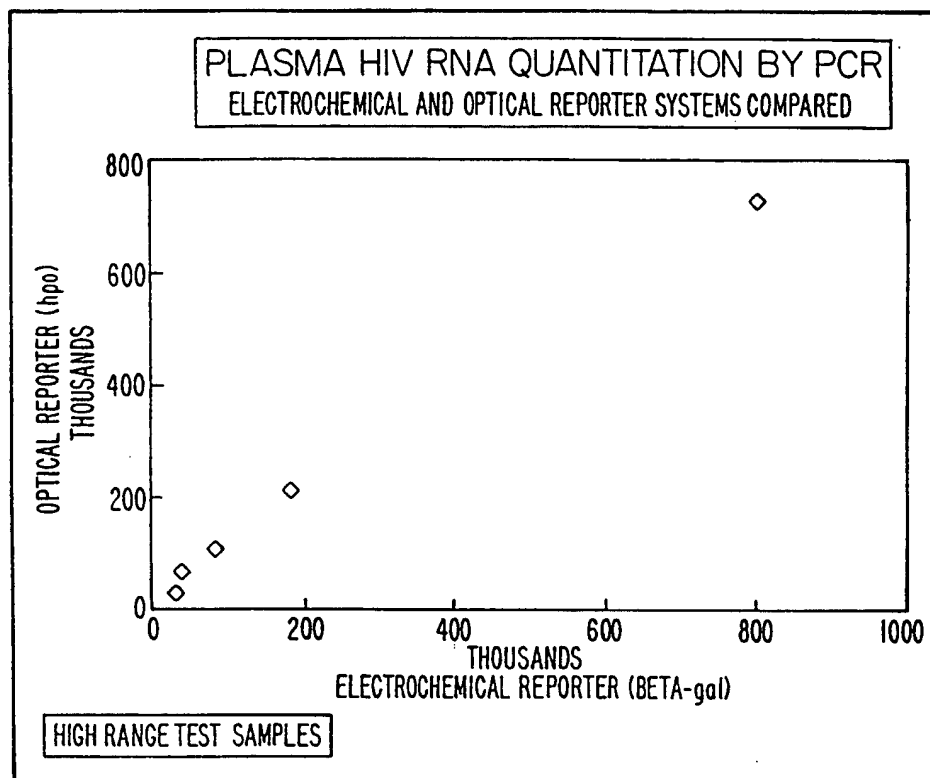


REGRESSION OUTPUT:

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NO. OF OBSERVATIONS	9
DEGREES OF FREEDOM	7
X COEFFICIENT (S)	1.063981
STD ERR OF COEF.	0.085985

FIG. 7.

FIG. 8.

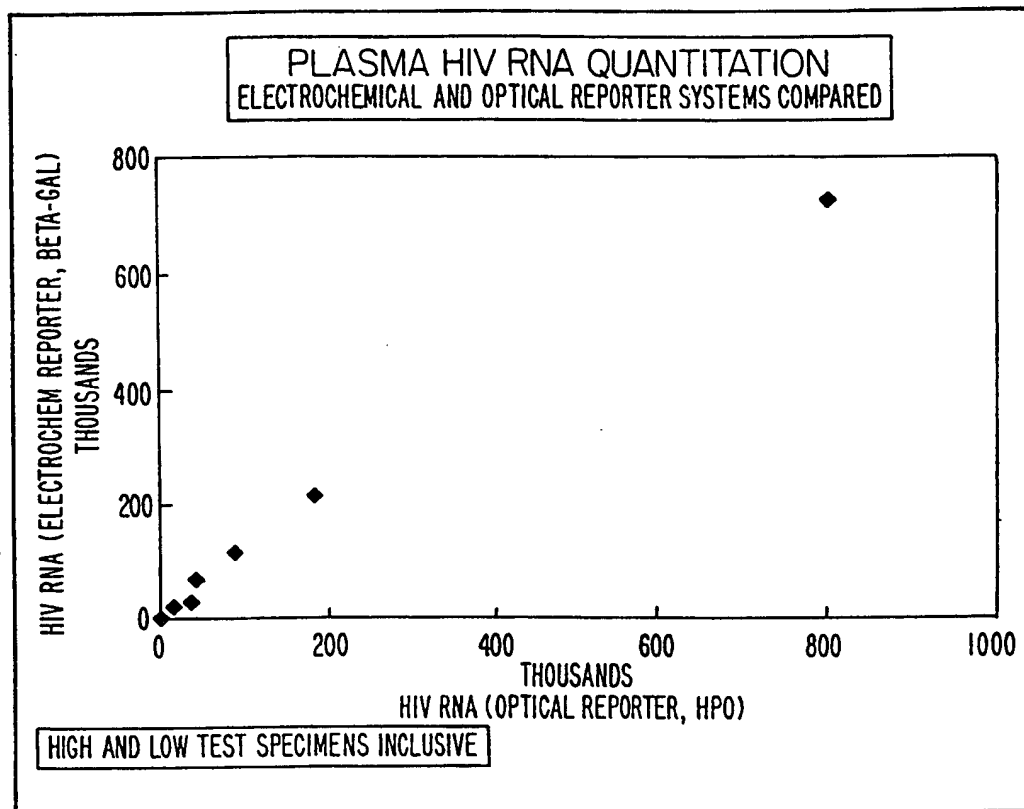


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X COEFFICIENT (S)	0.88036
STD ERR OF COEF.	0.037439

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FIG. 9.



REGRESSION OUTPUT:

CONSTANT	7285.766
STD ERR OF Y EST	17559.45
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NO. OF OBSERVATIONS	14
DEGREES OF FREEDOM	12

X COEFFICIENT	0.915861
STD ERR OF COEF.	0.022907

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/16714

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/00 G01N27/327 G01N33/543 C12Q1/68 C12Q1/42
C12Q1/34 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 840 893 A (HILL HUGH A O ET AL) 20 June 1989 see the whole document ----	1-25
Y	WO 94 29708 A (WOLLENBERGER ULLA ; HINTSCHE RAINER (DE); FRAUNHOFER GES FORSCHUNG) 22 December 1994 cited in the application see the whole document ----	1-25
X	US 5 391 272 A (O'DALY JOHN P ET AL) 21 February 1995 see abstract; figures ----	1
X	US 4 963 245 A (WEETALL HOWARD H) 16 October 1990 see abstract; examples ----- -/--	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

30 October 1998

Date of mailing of the international search report

13/11/1998

Name and mailing address of the ISA

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Authorized officer

Moreno, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/16714

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 16712 A (INST CHEMA UND BIOSENSORIK E V ;FELDBRUEGGE RAINER (DE)) 9 May 1997 see the whole document ---	1,11-13, 15,24,25
A	O. NIWA ET AL: "Small-volume voltammetric detection of 4-aminophenol with interdigitated array electrodes and its application to electrochemical enzyme immunoassay." ANALYTICAL CHEMISTRY., vol. 65, no. 11, 1993, pages 1559-1563, XP002082750 COLUMBUS US cited in the application see the whole document -----	1,11-13, 15,24,25

INTERNATIONAL SEARCH REPORT

Information on patent family members

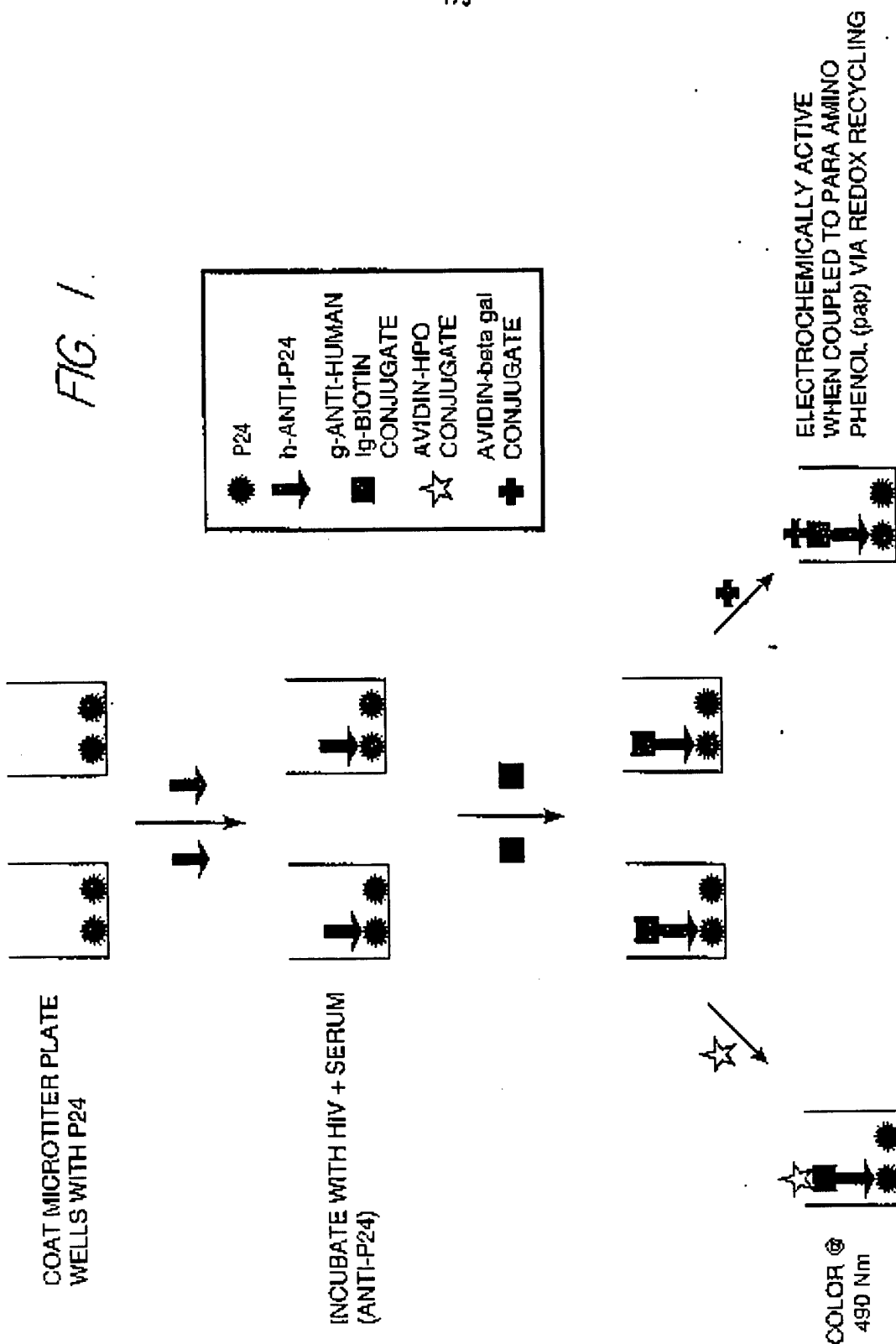
International Application No

PCT/US 98/16714

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Form PCT/ISA/210 (patent family annex) (July 1992)

FIG. 1



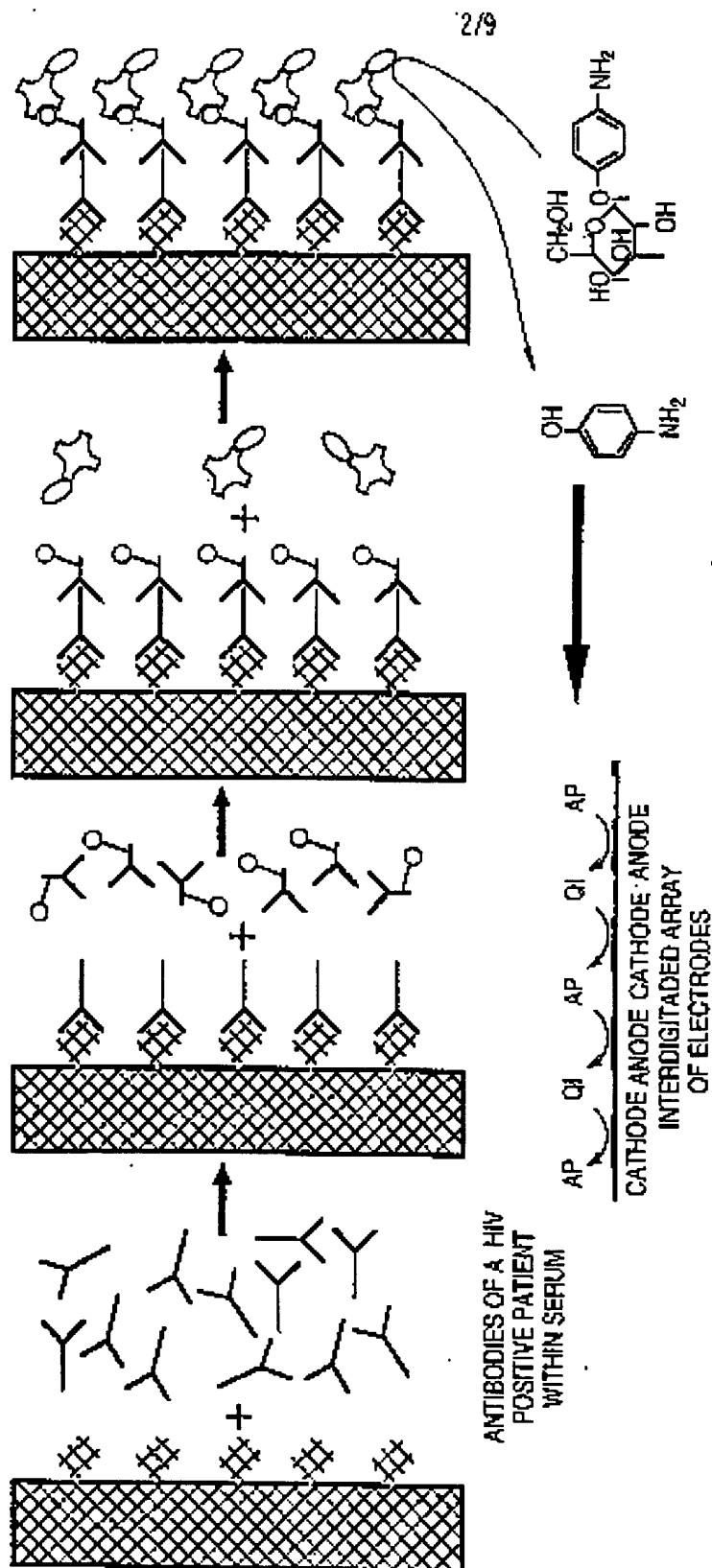
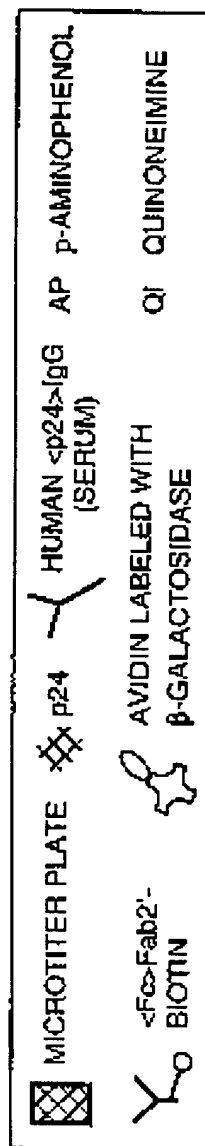


FIG. 2.



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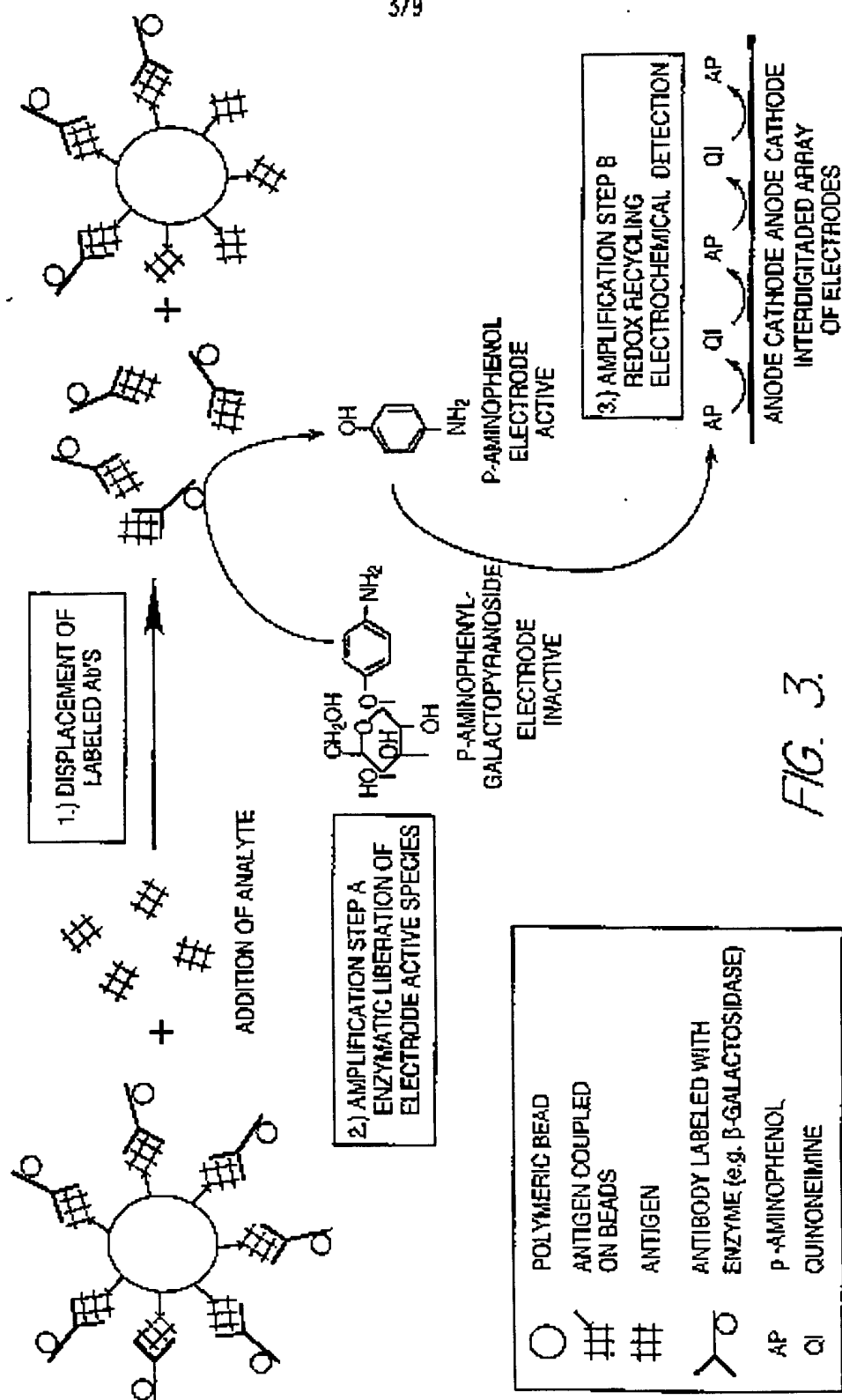
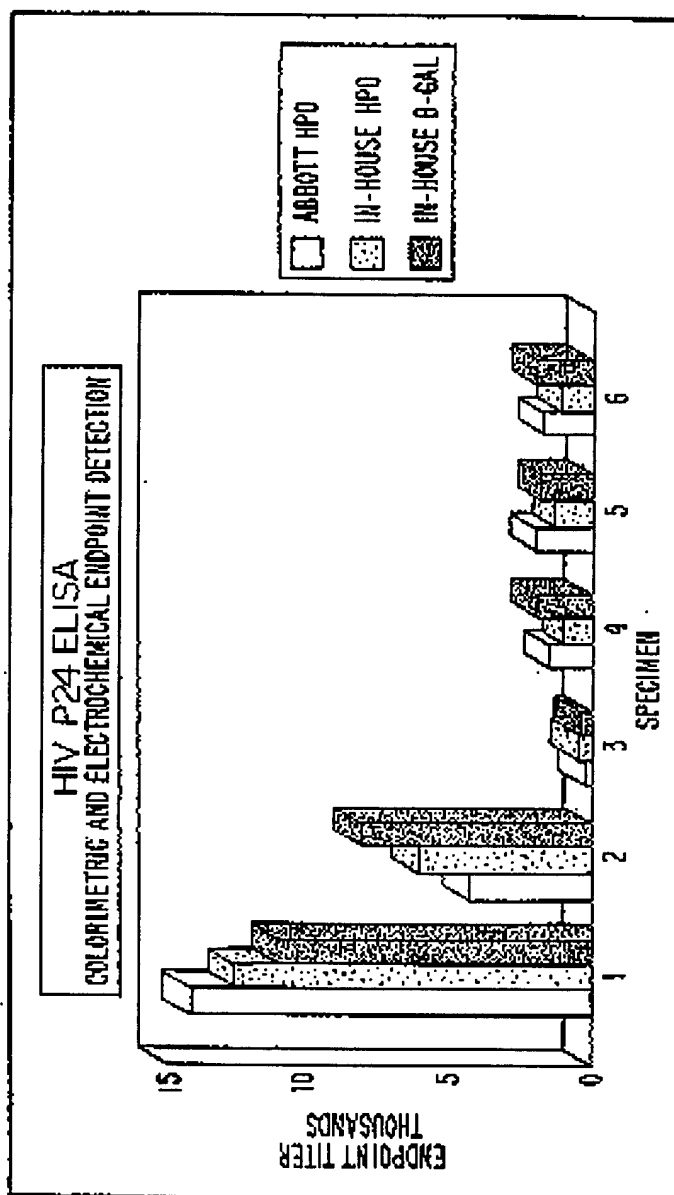


FIG. 3.

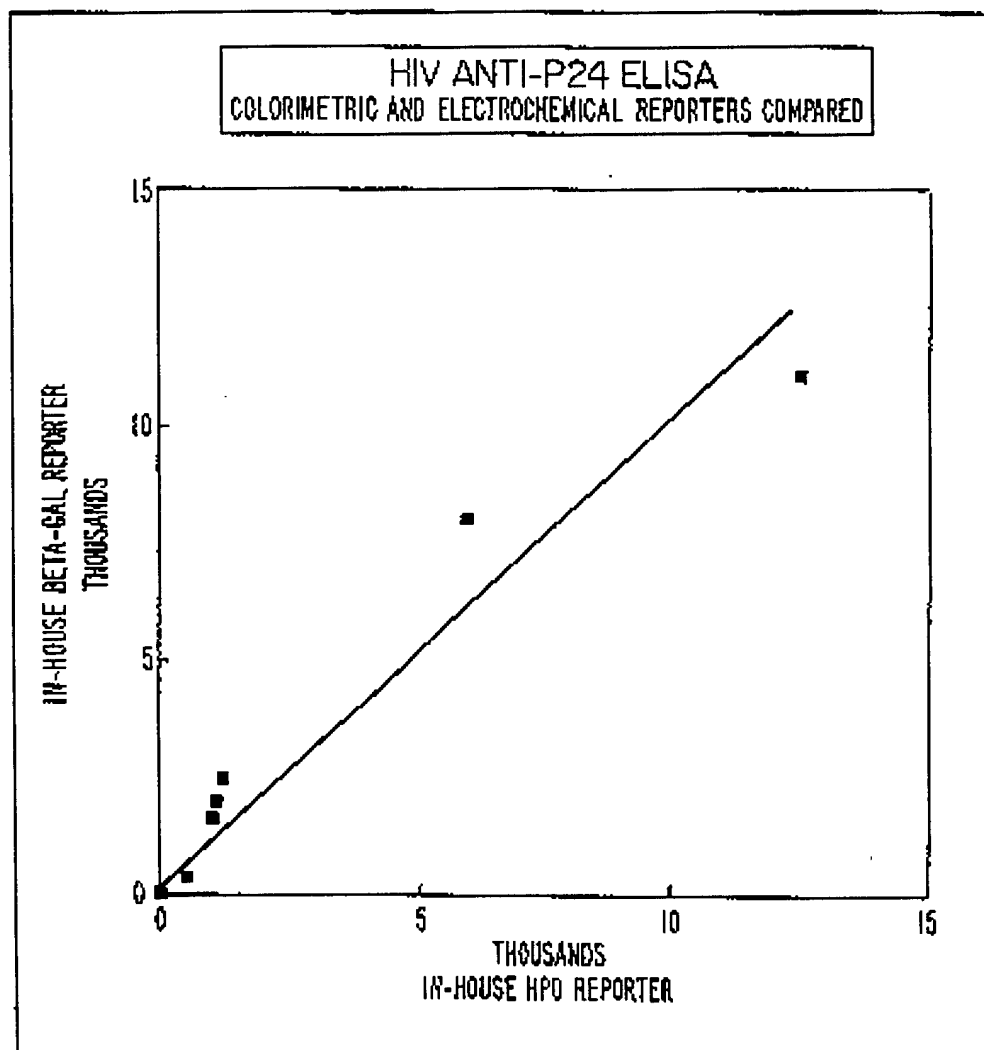
SPECIMEN NO.	COMMERCIAL ABBOTT	MIX AND MATCH HPO	MIX AND MATCH B-GAL
1	14000	12500	11000
2	4400	6000	8000
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4	1500	1000	2500
5	2000	1200	1700
6	1700	1100	2000
7	NEG	NEG	NEG
8	NEG	NEG	NEG
9	NEG	NEG	NEG
10	NEG	NEG	NEG

FIG. 4.



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FIG. 5.



REGRESSION OUTPUT:

CONSTANT	-390.639
STD ERR OF Y EST	1003.611
R SQUARED	0.944968
NO. OF OBSERVATIONS	10
DEGREES OF FREEDOM	8

X COEFFICIENT(S)	1.021692
STD ERR OF COEF.	0.087172

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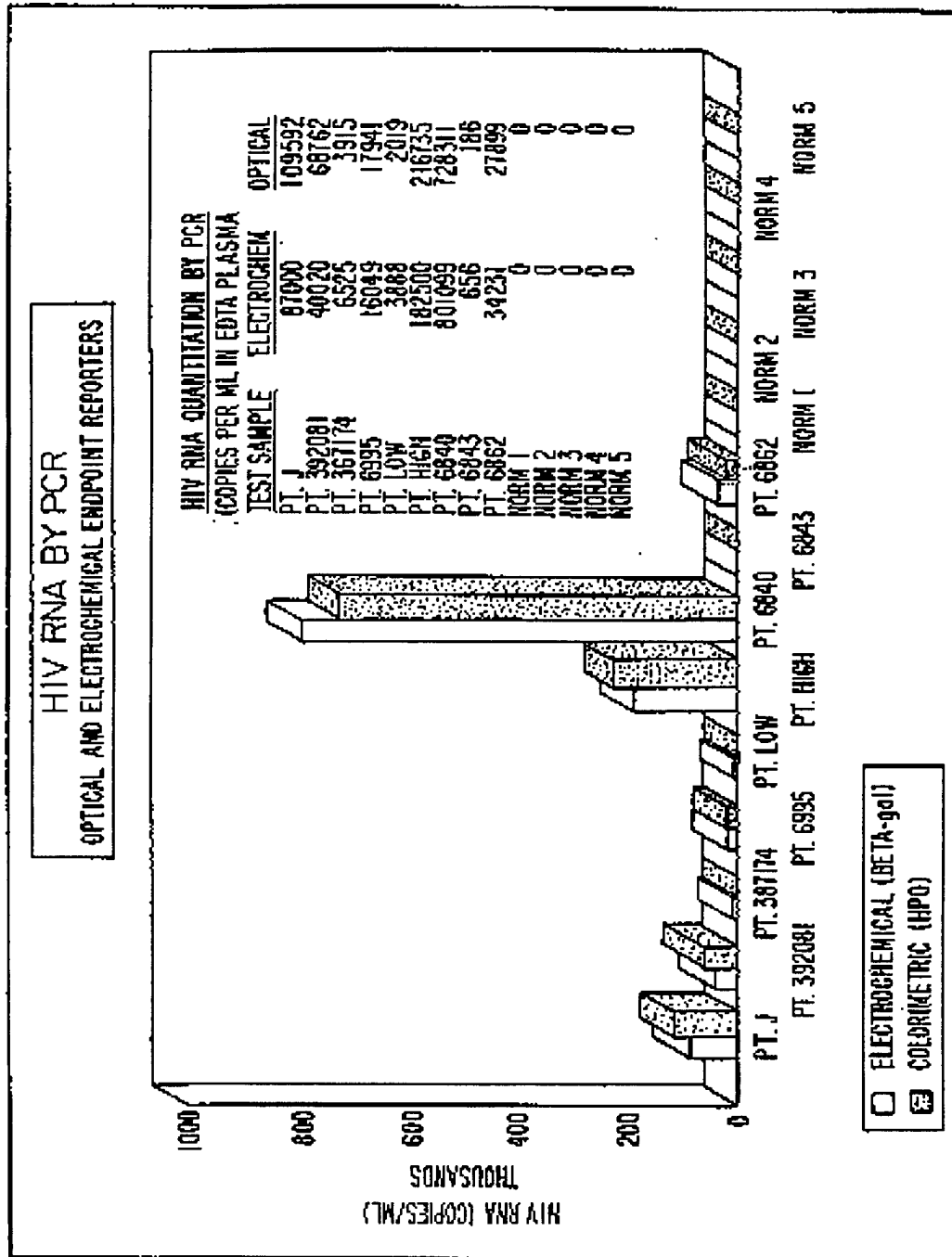
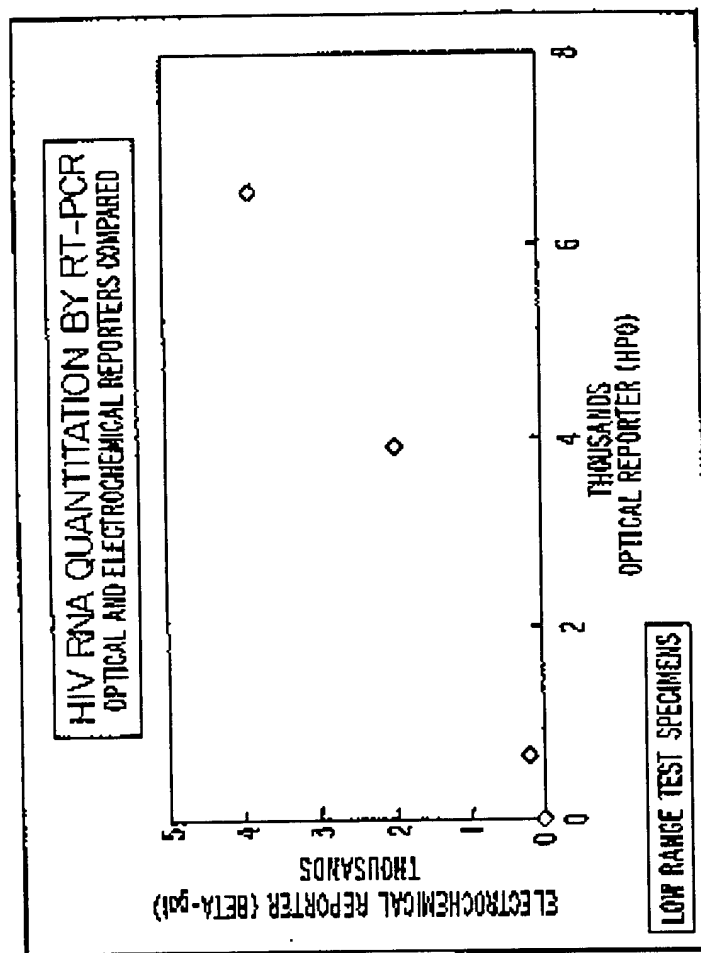


FIG. 6.



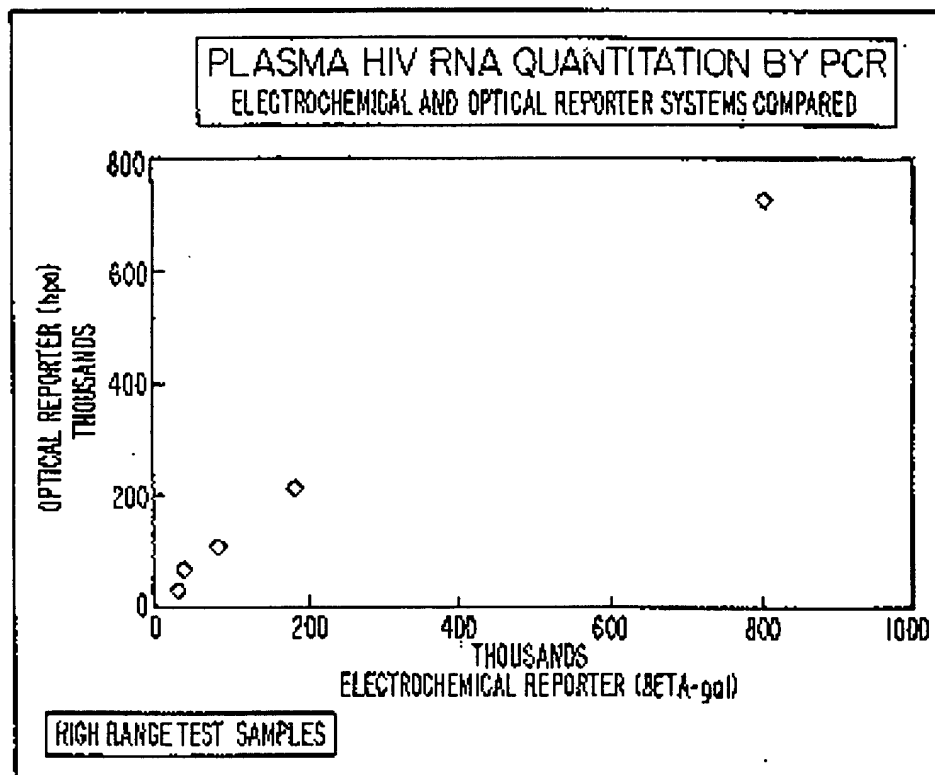
REGRESSION OUTPUT:

CONSTANT	-532.449
STD ERR OF Y EST	1315.275
R SQUARED	0.956282
NO. OF OBSERVATIONS	9
DEGREES OF FREEDOM	7

X COEFFICIENT (S)	1.063981
STD ERR OF COEF.	0.085985

FIG. 7.

FIG. 8.

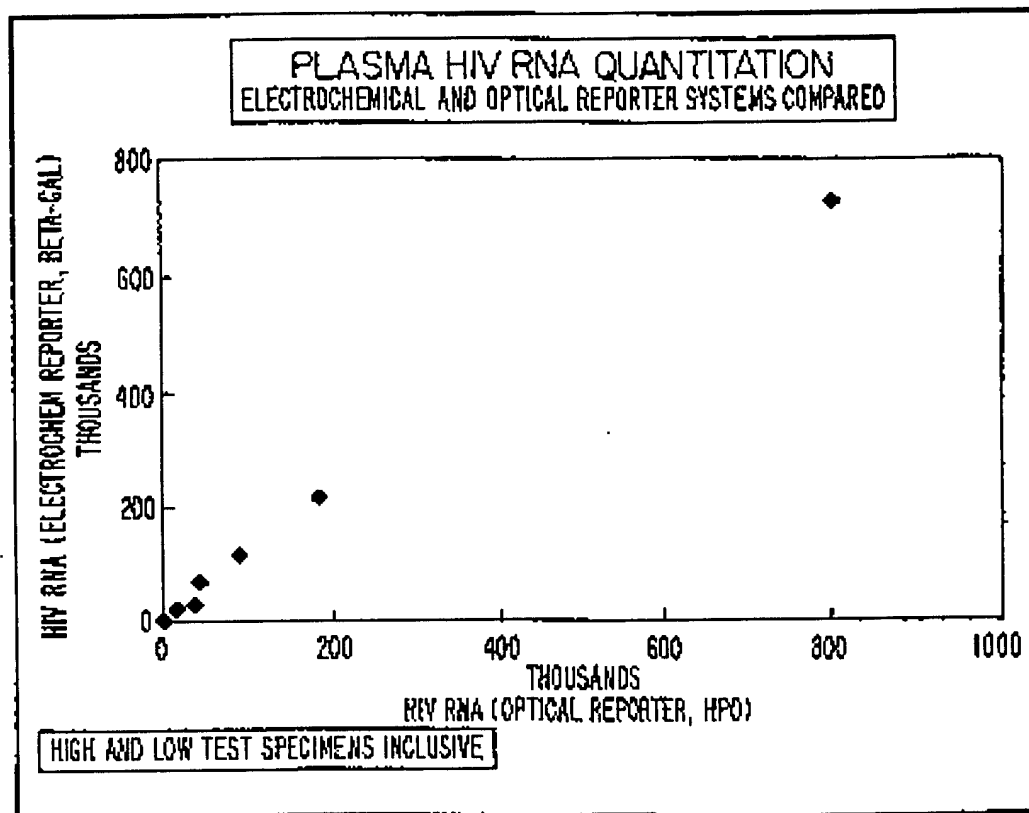


REGRESSION OUTPUT:

CONSTANT	28683.7
STD ERR OF Y EST	24357.31
R SQUARED	0.994604
NO. OF OBSERVATIONS	5
DEGREES OF FREEDOM	3
X COEFFICIENT (S)	0.88036
STD ERR OF COEF.	0.037439

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FIG. 9.



REGRESSION OUTPUT:

CONSTANT	7285.766
STD ERR OF Y EST	17559.45
R SQUARED	0.992549
NO. OF OBSERVATIONS	14
DEGREES OF FREEDOM	12

X COEFFICIENT	0.915861
STD ERR OF COEF.	0.022907

SUBSTITUTE SHEET (RULE 26)